IN THE SPECIFICATION

Please amend the specification as follows:

Page 1, lines 17-20:

Tuberculosis is the number one killer disease. Every year it kills largest number of people due to a single infectious disease. According to a World Health ~~Organisation~~ Organization Report (WHO) ~~reports~~ over 8 million cases of tuberculosis ~~is~~ are reported every year with over 2.9 million deaths ~~(Dolin et al., 1995).~~

Page 1, line 27, through page 2, line 7:

Over 70 species of mycobacteria are known, most are non-pathogenic for humans. Tuberculosis is caused by infection due to *M.tuberculosis*, with a few cases being caused by *M.bovis*. These organisms are genetically very close and called as ~~mycobacterium tuberculosis~~ *Mycobacterium tuberculosis* complex (MTC) organism. There is ~~other~~ over a dozen ~~of~~ other pathogenic mycobacteria, which causes tuberculosis like infection of lungs or other parts of the body. These organisms are called as mycobacteria other than tuberculosis (MOTT) or non-tuberculous mycobacteria (NTM). In the wake of AIDS epidemic these so called non tuberculous mycobacteria have become significant and are being isolated from large number of tuberculosis patients co-infected with HIV.

Page 2, lines 7 through 11:

Early tuberculosis often goes unrecognized in an otherwise healthy individual. The lack of simple, rapid and reliable tests that can specifically detect *M.tuberculosis* and other causative agents in a clinical specimen poses enormous ~~problem~~ problems for both individual patient management and implementation of appropriate infection control and public health measures.

Page 2, lines 12 through 18:

Classical methods of diagnosis include examination of a sputum smear under a microscope for acid-fast mycobacteria and a ~~x~~ X-ray of the lungs. However, in a vast majority of cases the sputum smear examination is negative for mycobacteria in the early stages of the

disease, and lung changes may not be obvious on a ~~x~~ X-ray until several months following infection. ~~Although staining~~ Staining of smear for acid-fast bacilli (AFB) takes less than two hours but lacks sensitivity and may be non-specific in some case~~.~~ ~~(Ebersole, L.L. 1992)~~. Moreover a positive result by AFB staining does not discriminate between the mycobacterium species.

Page 2, lines 19 through 25:

Currently the only absolutely reliable method of diagnosis is based on culturing *M.tuberculosis* from the clinical specimen and identifying it morphologically and biochemically. Culturing of *M. tuberculosis* and other related ~~organism~~ organisms is sensitive and specific but is cumbersome and may take 6-12 weeks while culturing on solid media and three to six weeks on liquid media, during which time a patient may become seriously ill and infect other individuals. Therefore, a rapid test capable of reliably detecting the presence of *M.tuberculosis* is vital for the early detection, treatment and management of the patient.

Page 2, line 26 through page 3, line 6:

Several molecular tests have been developed recently for the rapid detection and identification of *M.tuberculosis*. A commercial test, the Gen-Probe "Amplified Mycobacterium Tuberculosis Direct Test" has been evaluated by Abe et al and Miller et al. This test amplifies ~~M. tuberculosis~~ *M.tuberculosis* 16S ribosomal RNA from respiratory specimens and uses a chemiluminescent probe to detect the amplified product with a reported sensitivity of about 91%. Other commercial tests based on ligase chain reaction (LCR) (Abbott Laboratories), polymerase chain reaction (PCR) (Roche Diagnostics Systems, Eastman Kodak Co., Johnson & Johnson), Q-beta replicase (Gene Trak), and strand displacement amplification (Becton Dickinson). ~~are discussed in a review by Forbes.~~

Page 3, lines 7 through 12:

Other methods based on immunological detection of infection with *M.tuberculosis* by non-culture methods are latex agglutination, radioimmunoassay and enzyme linked immunosorbent assay, etc. Main drawback of these methods is their lack of sensitivity and or specificity (Kandival, G.V. et al., 1986; 1984; Yenez, M.A. et al., 1986). (Yenez, M. A., M. P. Coppola, D. A. Russo, E. Delaha, S.D. Chaparas, and H. Yeager Jr. 1986. *J.Clin. Micobiol.* 23: 822-825). Serological techniques may be useful in some clinical settings but this approach is limited in general due to poor sensitivity and or specificity. (Daniel, T.M. and S.M. Debanne. 1987) (Daniel, T. M., and S.M. Debannne, 1987. *Am. Rev. Res. Dis.* 135: 1137-1151).

Page 3, line 24, through page 4, line 8:

The discovery of the IS6110 insertion element (Cave et al., Thierry et al. 1990) (Eisenach, K. D., M. D. Cave, J, H. Bates and J. T. crawford. 1990 *J. Infect.Dis.*161: 997-981) (Thierry, D., A. Brisson-Noel, V.Levy-Frebault, S. Nguyen, J.Guesdon, and B. Gicquel. 1990. Thierry, D., Cave , M. D, Crawford, J. T, Bates, J. S, Gicquel, B. and Geusdon, J. L. 1989. *Nuc. Acid. Res.* 18: 188) and the belief that this element may only be present in *M.tuberculosis* complex (*M.tuberculosis*, *M.bovis*, *M.africanum* and *M. microti*) spawned a whole series of rapid diagnostic strategies (Brisson-Noel et al. 1991, Clarridge et al., al., Forbes et al., Hermans et al. 1990, Kolk et al. 1992, Kox et al. 1999, Zambardi et al.1993). (Brisson-Noel, A., C.Aznar, C.Chureau, S. Nguyen, C. Pierre, M. Bartoli, R. Bonte, G.pialoux, B. Gicquel, and G. Garrigue. 1991. *Lancet* 338: 364-366). These tests employ various techniques to extract DNA from the sputum. PCR is used to amplify IS6110 DNA sequences from the extracted DNA. The successful amplification of this DNA is considered to be an indicator of the presence of *M.tuberculosis* infection. U.S. Pat. Nos. 5,168,039 and 5,370,998 has been issued to Crawford et al. for the IS6110 based detection of tuberculosis. Another US patent No.5, 731,150 ~~have~~ has been granted to CIBA CORNING DIAGNOSTICS CORP (Gurpreet.S et.al 1998). European patent EP 0,461,045 has been issued to Guesdon .J.L for the IS6110 based detection of tuberculosis. The IS6110 element was reported to be present at ten, two, one, five and five copies in *M. tuberculosis*, *M. bovis*, M. bovis-BCG, *M.africanum* and *M.microti* respectively (Spargo et al.) .

Most reports using IS6110 and other PCR based detection of tuberculosis claim sensitivities of over 75% and specificities approaching 100%.

Page 3, lines 13 through 23:

The development of polymerase chain reaction (PCR) (Saiki et al. 1988). ~~(Saiki, R. K., D. H. Gelfand, S.Stoffel, S. J. Scharf, R. Higuichi, G.T. Horn, K.B. Mullis and H. A. Erlich, 1988. *Science.* 239: 487-491),~~ that allows DNA to be amplified and detected from small amounts of nucleic acid samples has made it possible to detect *M.tuberculosis* specific nucleic acids in clinical specimens. Some of the earlier reports were based on the detection of the 16S ribosomal RNA or its gene. Detection of *M.tuberculosis* and related organisms by first amplifying a portion of DNA using a primer conserved for all bacteria then using species specific probes to detect different species of mycobacteria. Major drawback of this method is that this is cumbersome, and takes over 24 hours to complete. Species specific probes that are used to detect different species sequence in the amplified product vary only in few bases and subsequent analysis of this amplified DNA by assays based on hybridization, if carried out under less that ideal conditions can lead to a false positive test.

Page 3, line 24 through page 4, line 8:

The discovery of the IS6110 insertion element (~~Cave et al.,~~ Thierry et. al. 1990) and the belief that this element may only be present in ~~M.tuberculosis~~ *M.tuberculosis* complex (*M.tuberculosis*, *M.bovis*, *M.africanum* and *M. microti*) spawned a whole series of rapid diagnostic strategies (Brisson-Noel et al., Clarridge et al., al., Forbes et al., Hermans et al., Kolk et al., Kox et al., Zambardi et al.). These tests employ various techniques to extract DNA from the sputum. PCR is used to amplify IS6110 DNA sequences from the extracted DNA. The successful amplification of this DNA is considered to be an indicator of the presence of *M.tuberculosis* infection. U.S. Pat. Nos. 5,168,039 and 5,370,998 has been issued to Crawford et al. for the IS6110 based detection of tuberculosis. Another US patent No.5, 731,150 have been granted to CIBA CORNING DIAGNOSTICS CORP (Gurpreet.~~S~~ et.al). European patent EP 0,461,045 has been issued to Guesdon, .J.L for the IS6110 based detection of tuberculosis. The

IS6110 element was reported to be present at ten, two, one, five and five copies in *M. tuberculosis*, *M. bovis*, ~~M. bovis BCG~~ *M. bovis BCG*, *M.africanum* and *M.microti* respectively. ~~(Spargo et al.).~~ Most reports using IS6110 and other PCR based detection of tuberculosis claim sensitivities of over 75% and specificities approaching 100%.

Page 5, lines 7 through 11:

Another fact which makes IS6110 an unsuitable target for the detection of tuberculosis is that some recent reports has showed that some *M.tuberculosis* isolates may altogether lack IS6110 sequence in its genome thus leading to false negative results. Studies on Asian isolates have reported that this sequence may be missing in at least some of the isolates (Yuen, L.K, et al. 1993) (Yuen, L.K. w., Ross, B.C, Jackson, K. M. and Dwyer, B. 1993. *J.Clin. Microbiol.*31: 1615-1618.).

Page 5, line 12, through page 6, line 7:

Another very important aspect of detection, differentiation and treatment of tuberculosis is the emergence of human immuno deficiency virus (HIV). Epidemiology and etiology of tuberculosis has undergone ~~sea~~ change since the rise of HIV, the causative agent for acquired immuno deficiency syndrome (AIDS). Incidence of tuberculosis has increased considerably since the emergence of AIDS (Bafica, ~~A~~.et al. 2003) ~~(Bafica A, Scanga CA, Schito ML, Hieny S, Sher A. 2003 J Immunol. Aug 1; 171(3): 1123-7).~~ Among AIDS deaths over 30% are due to tuberculosis. Since 1991 number of tuberculosis patients infected with HIV has increased from 3% to over 10%. Among AIDS patients only *M.tuberculosis* and *M.bovis* are not the only causative agents for tuberculosis. So called non-tuberculous mycobacteria have become significant pathogens in immunocompromised tuberculosis patients. Different laboratories have isolated other pathogenic mycobacteria called as non-tuberculous mycobacteria from clinical specimens derived from patients co-infected with HIV. Most important among them are *M.avium* and closely related group of mycobacteria ~~ie~~ i.e. *M.intracellulare* and *M.chelonae*. These organisms are known as ~~mycobacterium avium intracellulare~~ *Mycobacterium avium-intracellulare* complex (MAI complex) organisms. MAI complex of organisms presents

symptoms that are indistinguishable from tuberculosis. They are responsible for pulmonary as well as disseminated form of disease in a large numbers of patients especially those infected with human immuno-deficiency virus (HIV). *M.avium* alone has been isolated from ~~upto~~ up to 30% of clinical specimens from ~~Pulmonary~~ pulmonary tuberculosis patients and at even higher number from disseminated tuberculosis patients. ~~*M.kansassi*~~ *M.kansasii* and *M.scrofulaceum* are other non-tuberculous mycobacteria that have been isolated from considerable number of AIDS patients with tuberculosis. Other non-tuberculous mycobacteria are also being isolated from clinical specimen derived from AIDS patient. Reason for fewer isolation of non-tuberculous mycobacteria may be non availability of simple, accurate and reliable tests to isolate and differentiate different types of non-tuberculous mycobacteria. These findings suggest that the non-tubercular mycobacteria have become significant etiological agents in the wake of emergence of AIDS.

Page 6, lines 8 through 12:

~~This information that~~ IS6110 is not specific for *M.tuberculosis* and may be absent in many isolates ~~together with the fact that~~ and other non-tubercular mycobacteria are the causative agent for tuberculosis especially in patients co-infected with HIV. It is clear from published reports that no existing technique based on IS6110 and other target sequence provides a level of confidence needed in a clinical diagnostic test.

Page 6, lines 13 through 25:

This accentuates need for change in the approach of detection of tuberculosis. This calls for evaluation of new targets that ~~is~~ are able to detect all pathogenic mycobacteria in a clinical specimen instead of detecting only *M.tuberculosis* complex group of organisms. Ideally there should be a diagnostic method that instead of detecting only *M.tuberculosis* complex group of bacteria should detect all pathogenic mycobacteria including non-tubercular mycobacteria in a clinical specimen. After detection of different pathogenic mycobacteria in a clinical specimen different types of pathogenic mycobacteria can be differentiated into different species of mycobacteria by PCR-RPLF method as described in this assay. Those patients infected with

NTM alone or NTM together with *M.tuberculosis* complex group of organism will give a quick reference for possible co-infection with HIV and thus could be a good parameter to access HIV infection and spread in the population. Not many such tests are available that can detect pathogenic mycobacteria in a clinical specimens as well as differentiate them.

Page 6, lines 26 through 31:

Mycobacterium tuberculosis Direct Test has been evaluated by Abe et al and Miller et al. This test amplifies ~~M. tuberculosis~~ *M.tuberculosis* 16S ribosomal RNA from respiratory specimens and uses a chemiluminescent probe to detect the amplified product with a reported sensitivity of about 91%. This test is complex, takes over 24 hours to complete and uses probes to identify different mycobacteria vary only in few bases which yields false positive result if done in even slightly less stringent condition.

Page 7, lines 7 through 21:

A major weakness of currently available PCR based assays for detection of mycobacteria is the lack of a method of nucleic acid extraction that is simple, efficient and ensures safety to the user. Lysis of mycobacteria and purification of nucleic acid from clinical specimen without co-purifying impurities, which are known to be present in most clinical specimens, is a crucial step in a PCR based assay. A major drawback of the published protocols is that most methods used for extracting nucleic acids cannot be easily used for all types of specimens. Any nucleic acid extraction that necessitates a tedious and inefficient DNA purification will decrease the speed and sensitivity of the test. Additionally, having to carry out a different extraction procedure on different types of samples also makes the whole process expensive and slow. Operator safety is also a major concern when handling samples containing live *M.tuberculosis*. It was found that after careful analysis of different DNA extraction procedures described earlier that they were either highly inefficient or unable to remove impurities that are generally present in most clinical specimens (Boom, et. al. 1990) ~~(Boom, R. C .J. A.sol, M. M. salimans, C. L. jansen, P. M. E. Wertheim-Van Dillen, and J.Van der Noorda.1990. J.Clin.Microbiol. 28: 495-503)~~. A simple,

efficient and robust method of nucleic acid extraction from various clinical specimens was thus required to ensure sensitivity and reproducibility of a PCR based assay.

Page 8, lines 15 through 26:

Present invention relates to detection of pathogenic mycobacteria in clinical specimens such as sputum, cerebrospinal fluid, gastric lavage and tissue biopsies etc. Novelties of the invention ~~lies~~ lie in novel stretch of DNA that lies in the intergenic region between methyl mycolic acid synthase genes mmaA1 and mmaA2 and the flanking region in mmaA1 and mmaA2 genes. This test uses a pair of oligonucleotide primers that specifically amplifies the target DNA from the clinical specimens. The invention describes a method of DNA extraction from clinical specimen, which is safer and yields more DNA from clinical specimens than the existing methods. Present invention also describes DNA amplification method that result in specific amplification of the target amplicon without use of expensive reagents thus making the test economical. Present invention elucidates a method for differentiation of different species of pathogenic mycobacteria in the clinical specimen by restriction fragment length polymorphism (RFLP) analysis of the amplified PCR product.

Page 8, lines 34 through 37:

Fig. ~~Figure~~ 2. Sequence of mmaA2 and mmaA1 gene with an intergenic region of 166 base pair~~d~~ (shown in lower case. Location of forward A, ~~sequence~~ SEQ ID NO:1 and reverse primer D, ~~sequence~~ SEQ ID NO:2. ~~Both primer sequence is underlined and italisized italicized~~. Positions of both primer sequences are underlined.

Page 9, lines 6 through 11:

Fig. ~~Figure~~ 3. PCR amplification of different mycobacterial genomic DNAs with primers A and D (lanes 1-15); 1. *M.avium* 2. *M.bovis* 3. *M.chelonae* 4. *m.fortutitum* 5. *M.intracellulare* 6. ~~*M.kansassi*~~ *M.kansasii* 7. *m.phlei* 8. 100 bp DNA ladder 9. *M.marinum* 10. *M.scrofulaceum* 11. *M.smegmatis* 12. *M.szulgai* 13. *M.tuberculosis* and 14. negative control. AD indicates 363 bp-amplified product.

Page 9, lines 25 through 30:

The objective of this work has been to develop a comprehensive technique that allow for rapid, safe and specific detection of tuberculosis causing mycobacteria. The limiting ~~step~~ steps in any PCR based diagnosis are DNA extraction from clinical specimen, design of PCR primer that can specifically amplify target sequence and developing a PCR condition that will allow only specific amplification of the target sequence. A serious limitation of available tests is that they detect but could not differentiate various species of mycobacteria.

Page 9, line 31, through page 10, line 3:

Various available protocols describe different methods of nucleic acid extraction and mix of reagents to lyse mycobacteria and purify nucleic acid from a clinical specimen. In various methods lysis is achieved by treatment of the specimen with alkalis, organic solvents, chaotropic agents, detergents or a mix of them. Several of the simpler ~~method claims~~ methods claim to achieve the lysis by simple boiling in alkali, PCR buffer or even in plain water. Although these methods are simple and work generally well on pure culture, ~~but~~ they are not so useful for a clinical specimen. The generally prevailing notion that PCR reaction is robust and the nucleic acid liberated by crude lysis methods can be used directly in a PCR reaction is not true. These methods are simple to use but often fail to kill all mycobacteria present in a clinical specimen and thus could be hazardous for the user. Such preparations are reported to contain many impurities that can easily inhibit a PCR reaction. It has been observed that such preparation may not result in amplification even on diluting the DNA several folds. The fact that extraction of clean DNA is of utmost importance for success of a PCR based assay.

Page 10, lines 4 through 23:

The inventors have carefully optimized all steps in nucleic acid purification and developed a method that is simple, robust, and efficient and ~~ensure~~ ensures complete safety for the operator. Further the treatment of the dirtier specimen like sputum by mild alkali and a mucolytic agent helps to remove many contaminating agents and results in cleaner nucleic acid preparation. This ~~steps~~ step also helps in removing other contaminating organisms present in

dirtier samples like sputum and gastric lavage etc. Since ~~Sputum~~ sputum is the most commonly collected and submitted clinical specimen for pulmonary tuberculosis and is known to contain several contaminants that is potent inhibitor or PCR reaction~~. The~~ , the modified lysis buffer developed in the prevent invention uses a strong chaotropic agent, ie, ~~i.e~~ guanidinium isothiocyanate. This helps to inactivate all mycobacteria present in a clinical specimen, lyse tough mycobacterial cell and denature and remove proteins ~~thus results~~ resulting into cleaner preparation of DNA (Table 3) and also ~~ensure~~ ensures safety for the operator. By heating specimen in our lysis buffer even the toughest cells and objects like spores and baculovirus polyhedra are lysed easily. An earlier report by the same group has revealed use of guanidinium isothiocyanate for lysis and purification of nucleic from tough materials like baculovirus polyhedra and mycobacteria (Das et.al 1996) and (Bose.~~M~~ et al 1998).

Page 11, lines 5 through 30:

Another advantage of using this reagent is that most proteins are denatured in this buffer leading to through lysis of the mycobacteria present in the specimens. Other method also describe using this reagent (Gurpreet, ~~S~~ et al. 1980). The present method is different from them in several ways. The lysis buffer in the present method has a composition that ~~accomplish~~ accomplishes more through lysis, does better deproteination and ~~help~~ helps precipitate even minute amount of DNA. This results in cleaner DNA preparation with improved yield. Instead of using guanidinium isothiocyanate-tris-phenol for lysis the lysis buffer of the present invention contain detergent N lauryl sarcosyl, 200 mM NaCl and 10 mM 2' mercaptethanol together with 4M guanidinium isothiocyanate. Phenol being ~~an~~ extremely explosive and thus hazardous is not included in the lysis buffer. These modifications have made lysis buffer of the present invention complete and more potent. Detergent helps in solubilization of cell wall lipid and of protein and thus result in complete lysis of the mycobacterial cell wall, which is rich in different types of complex lipids. Use of NaCl helps in precipitation of nucleic acid present at minute amount and thus gives approximately 1.4-1.5 fold better DNA yield than the method described by Gurpreet et al. This is crucial especially when dealing with a clinical specimen which has fewer numbers of mycobacteria per ml of sample. Mycobacterial cells are inactivated and lysed by heating the

digested and decontaminated sample in lysis buffer at 85°C for 20 min. This is safe, as compared to boiling as described in several methods is unnecessary and may result in popping of the cap or bursting of some tubes. The lysate is extracted once with alkaline phenol. It has been noticed that deproteination by extraction with alkaline phenol is not unnecessary as claimed by many protocols. This simple step leads to removal of all proteins including the ones tightly bound to DNA and thus leads to cleaner nucleic acid preparation. This increases reproducibility of the assay especially when dealing with dirtier samples like sputum and gastric lavage. Nucleic acid is precipitated from the aqueous phase with equal volume of iso-propanol.

Page 11, line 31, through page 12, line 15:

In the next step a set of oligonucleotide primer was designed that was specific for pathogenic mycobacteria. Care was taken to ensure that this sequence is absent in other pathogenic organisms or in human being since presence of such organisms and human cells cannot be ruled out in clinical specimens. A gene cluster mmaA1-mmaA4 accession numbers MTCY20H10.23c- MTCY20H10.26c of mycobacteria ~~(fig1)~~ (Fig. 1) was employed for this purpose. This gene cluster contains four genes separated by three spacer regions of various lengths ~~(fig1)~~ (Fig. 1). These genes methoxy-mycolic acid synthases are responsible for synthesis and modification of complex terminal mycolic acids present in pathogenic mycobacteria. These mycolic acids are implicated in pathogenicity of mycobacteria. Forward primer A, ~~sequence ID number3~~ SEQ ID NO:3 is located from 1-9 bases in mmaA2 gene, 11 bp of this oligonucleotide primer lies in the 167 bp spacer region between the genes mmaA2 and mmaA1. Reverse primer D, ~~sequence ID number 4~~ SEQ ID NO:4 is located from 688-705 bases in the mmaA1 gene Fig 1 and ~~fig2~~ Fig. 2. These primer sequences were designed using the software Primer Select (Lasergene, DNASTAR) and do not show homology with sequences of organisms other than *M.tuberculosis* and *M.bovis*.

Page 12, lines 16 through 27:

Another approach was also taken to ensure that this primer sequence is specific to pathogenic mycobacteria. The oligonucleotide sequence was converted to amino acid (peptide)

and compared to gene complement of many pathogenic organisms and human using a software Genome Calculator developed at this institute (Institute for Genomics and Integrative Biology). This software converts a DNA sequence into amino acid (peptide) and compares it with all sequences available in the database by converting them into library of short peptides. This software found primer sequences to be specific to *M.tuberculosis* and *M.bovis* and not to be present in any of the 24 other pathogenic organisms or human whose whole genome sequence was available in the databases. PCR with the genomic DNA of all pathogenic mycobacterium tested resulted in amplification using these primers while PCR using the genomic DNA of non-pathogenic mycobacteria did not resulted in amplification Fig. 3 and ~~Table1~~ Table 1.

Page 12, line 28, through page 13, line 5:

Table 1: PCR amplification of AD from different pathogenic and non-pathogenic species of mycobacteria

Mycobacterial Species	Strain	Pathogenic/nonpathogenic	PCR result
M.avium	ATCC	Pathogenic	positive
M.bovis	ATCC	Pathogenic	positive
M.chelonae	ATCC	Pathogenic	Positive
M.fortuitum	ATCC	Pathogenic	Positive
M intracellulare	ATCC	Pathogenic	Positive
~~*M.kansassi*~~ *M.kansasii*	ATCC	Pathogenic	Positive
M.marinum	ATCC	Pathogenic	Positive
M.phlei	ATCC	Non- pathogenic	Negative
M.smegmatis	ATCC	Non-pathogenic	Negative
M.scrofulaceum	ATCC	Pathogenic	Positive
M.szulgai	ATCC	Pathogenic	positive
M.tuberculosis	ATCC	Pathogenic	positive
M.xenopi	ATCC	Pathogenic	positive

Page 13, lines 6 through 13:

After design of the specific primer next critical step was to design and develop a PCR condition that will specifically amplify only the desired target. This is crucial since PCR in sub-

optimum condition result in nonspecific amplification of other stretch of DNA closely resembling the desired target in size Gurpreet et al. This in turn leads to less amplification of the desired target sequence and thus reduced specificity and sensitivity. Unlike the primers used by Gurpreet et, al. in their invention our primers do not ~~results into~~ result in amplification of a band of approximately same size from non-pathogenic mycobacteria even under sub optimal condition (Fig. 3).

Page 13, line 22, through page 14, line 6:

Further the cycling conditions were modified which would discourage annealing of primer at sites other than desired. This was achieved if the initial few cycles of the PCR were undertaken at higher annealing temperature than the calculated melting temperature of the oligonucleotide primer and then gradually reduce annealing temperature in each cycle and do rest 25 cycles at the optimum annealing temperature. Once the specific amplicon is established in the initial cycles, they do not allow nonspecific products compete with them in the later cycles. This type of cycling condition is called as touch down ~~Down~~ PCR and helps specific amplification without use of expensive reagents. This measure helps to make the test more cost effective and easy to use.

Page 14, lines 7 through 16:

Detection of the amplified PCR product is the next step in an assay. Amplified PCR product can be detected in several different ways. Electrophoresis on agarose or polyacrylamide gel is the most common and simple way of detecting an amplified PCR product. Use of agarose gel is simpler than polyacrylamide gel electrophoresis, or DNA ELISA, which is cumbersome, takes longer to detect and ~~need~~ needs more expertise. Polyacrylamide gels are less sensitive as compared to agarose gels since polyacrylamide quenches the ethidium bromide dye used for detection. Besides, acrylamide is a potent neurotoxin and thus potentially hazardous for the user. A horizontal agarose gel electrophoresis method was used for resolution of PCR product. The amplified product is detected on a short wave UV transilluminator.

Page 14, line 20, through page 15, line 8:

This PCR based detection method not only meant for detection of different types of pathogenic mycobacteria in a clinical specimen but can be used to differentiate them in various species using the restriction fragment length polymorphism (RFLP) analysis of the PCR amplified fragment. RFLP is a very powerful tool for different species and strains of a species. It is based on the fact that each DNA has site for one or more restriction endonucleases. These sites are recognized precisely by class II restriction endonucleases derived from different bacteria. In natural course of evolution of an organism one or more of these sites are modified or lost. Therefore restriction with the same enzyme of a stretch of DNA results in polymorphism in the fragment length among different species of organisms and thus serve as an efficient tool for differentiation and epidemiology. AD is a suitable candidate for PCR-RFLP analysis of different species of mycobacteria since this stretch has portion of two genes has an intergenic region of 167 bp. This DNA stretch has two sites of several restriction endonucleases separated by several bases that will yield fragments in the size range that is suitable for analysis by polyacrylamide gel electrophoresis ~~table~~ Table 2 (~~figures~~ Figs. 4, 5 and 6). These sites lie in intergenic as well as in the gene mmaA1 (~~figures~~ Figs. 4, ~~fig~~ 5 and ~~fig~~ 6). Restricted product can be easily separated on 10-12 % polyacrylamide gel for RFLP mapping of different species. Presence of an intergenic region of 167 bp combined with well-laid out sites for some common restriction endonuclease make AD a suitable candidate for differentiation of different species of pathogenic mycobacteria by PCR-RFLP analysis.

Page 15, line 11, through page 16, line 9:

The PCR reagents were made and dispensed in a clean room dedicated to PCR reagent preparation. No specimen, culture or purified DNA was ever introduced into the PCR mix room. Target DNA was added to the PCR mix in a separate room that has never been exposed to amplified DNA. Amplification was carried out in a MJ mini thermal cycler (M J Research) using 200-µl thin walled tubes with attached individual caps (Axygen). The first solution added to the PCR tube contained 2.0 µl of 10 x PCR buffer (100 mM Tris.Cl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 2.0 µl of dNTP mix (2.0 mM each of dATP, dGTP, dTTP and dCTP), 1.0 µl of primer

SEQ ID NO: 5, 5 TGGATCCGTTGACCATGAGGTGTAATG 3 (5 picomoles/µl), 1.0 µl of primer SEQ ID NO: 6, 5 GGAATTCCACTACGCACGGACTCTC 3 (5 picomoles/µl) and 0.2 µl Taq polymerase containing 1 unit of enzyme and 11.8 µl of water. The PCR tubes were taken to the sample preparation room and 2.0 µl of the DNA was added to it. The tubes were mixed well by tapping and were run on touch down ~~Touch Down~~ PCR program (~~fig:~~ Fig. 7). This ~~programme~~ program had one initial denaturation at 95 °C for 3 min. Initial denaturation step was followed by 14 cycles of touch down ~~Touch Down~~ containing one denaturation at 94 °C for 45 sec, one annealing beginning at 70 °C for 45 sec with a decrement of 0.8 °C in each touch down ~~Touch Down~~ cycle and one extention at 72 °C for 1 min. This was followed by 25 cycles of normal cycling containing one denaturation at 94 °C for 45 sec, one annealing at 58 °C for 45 sec and one extention at 72 °C for 1 min. On completion of PCR the tubes were taken to another room for the analysis of amplified PCR product.10 µl of the reaction was loaded on the 2.0% agarose gel, another 10 µl of the reaction was saved for PCR-RFLP analysis wherever required.

Page 16, lines 13 through 26:

Out of these 74 specimens that were positive by acid fast smear method, 68 were also positive by PCR. Four of them were positive by smear but negative by PCR. Smear result of all these patients had scanty report by acid fast microscopy. Besides duplicate samples from these patients were positive by PCR. Two specimens coming from the same patients were positive by ~~Smear~~ smear but negative by PCR. Upon spiking of reaction containing DNA from these specimens with purified DNA these samples were found to contain inhibitors of PCR. These specimens ~~when~~ were treated again with the lysis buffer and precipitated with iso-propanol yielded amplification. Thirty-one patients were negative by ~~Acid~~ acid fast microscopy but positive by PCR. Clinical history of all these patients showed that they were positive for mycobacteria by smear method and were undergoing treatment and being smear negative was due to low bacillary load in these ~~specimen~~ specimens. Remaining thirty-seven specimens were negative by both smear and PCR method. When their clinical report were examined they were found to be negative by other clinical parameters and had come to the hospital on the basis of

preliminary symptoms like fever and cough.

Page 17, lines 18 through 22:

(f) developing a PCR amplification process for specific amplification of SEQ ID No.4 of (d) said process comprising using the specific oligonucleotide primers ~~of~~ designed and synthesized in step (e) for detecting presence of pathogenic mycobacteria in the clinical specimens and

Page 17, lines 23 through 26:

(g) ~~analysing~~ analyzing the amplified PCR product by restriction fragment length polymorphism (RFLP) analysis for differentiation of the species of the pathogenic mycobacterium for a quick assessment of HIV co-infection.

Page 19, lines 10 through 15:

Still another embodiment of the present invention relates to the high yielding amplification of DNA in step (f) is achieved by the modified Touch ~~down~~ Down PCR cycling conditions said conditions comprising steps of initial high annealing temperature in the range of about 62-72 °C followed by lowering of temperature in the range of about 0.2 - 1 °C per PCR cycle for the first 10-25 cycles, which is the ~~touch down~~ Touch Down step to an optimum annealing temperature of about 56-62 °C for another 30 PCR cycles.

Page 19, lines 16 through 20:

One more embodiment of the present invention relates to high yield amplification of DNA is achieved by modified Touch Down PCR cycling conditions, said ~~constions~~ conditions comprising steps of initial high annealing temperature is about 70°C followed by lowering temperature is about 0.8 °C per PCR cycle for about first 14 cycles to about 58 °C for another 25 PCR cycles.

Page 21, lines 8 through 12:

Cerebrospinal fluid (CSF) is considered to be generally sterile and ~~do~~ does not need digestion and decontamination. 1-2 ml of cerebrospinal fluid (CSF) was transferred to a micro

centrifuge tube (MCT) and spun at 12000g for 3 minutes in a micro-centrifuge (Eppendorf, A.G Germany) .The pellet was washed with 0.067M phosphate buffer pH 7.0 and resuspended in 300~~u~~µl sterile distilled water. Whole was used for PCR.

Page 21, lines 14 through 21:

Acid–fast staining was done using basic fuschin dyes by staining procedure of Zeihl-Neelsen. From the mucoid part of sputum a small part was smeared in 1x2 cm. ~~Area~~ area. Smear was briefly heat fixed, flooded with basic fuschin dye and heated briefly over Bunsen burner. De-stained with acid alcohol 3% sulfuric acid in 95% ethanol. Slides were washed with distilled water and counter-stained with methylene blue (0.3% methylene blue chloride) for 1-2 minutes. Rinsed with water and air-dried. Slides were examined under oil immersion objective at 400x with a binocular microscope (Zeiss, Germany). Smear were scored as per WHO guidelines.

Page 22, lines 6 through 22:

Two ~~Oligonucleotide~~ oligonucleotide primers that amplify a portion of an essential gene of pathogenic mycobacterium were designed using Primer select software (DNASTAR software from LASERGENE INC). Methyl mycolic acid synthase is a cluster of four genes, mmaA1-mmaA4. These genes are involved in synthesis and modification of mycolic acids and is reported to be present only in pathogenic mycobacteria. 11 bp of forward primer lies in the intergenic region between mmaA1 and mmaA2 genes, while reverse primer is located in the methyl mycolic acid synthase 1 gene (mmaA1). These primers were checked for specificity to mycobacterium using the software GENOME CALCULATOR developed by Bio-informatics division at this Centre. Forward oligonucleotide primer, ~~sequence ID~~ SEQ ID NO:5 is 27 bp long. Reverse primer ~~sequence ID number~~ SEQ ID NO:6 is 25 base pairs long. Forward primer A is located from 1-9 bases in mmaA2 gene, 11 bp of this oligonucleotide primer lies in the 167 bp spacer region between the genes mmaA2 and mmaA1. Reverse primer D, ~~sequence ID~~ SEQ ID NO:2, is located from 688-705 bases in the mmaA1 gene Figures 1 and 2. Primer sequence has an overhang of seven base pairs at the 5' containing site for Bam HI restriction endonuclease. Oligonucleotide primer D also has a seven-bp overhang at the 5' end-containing

site for EcoRI. These primers specifically amplify a 373 base pairs portion designated as AD of an essential gene of pathogenic mycobacterium.

Page 22, line 23:

Forward primer ~~Sequence ID number~~ SEQ ID NO:5

Page 23, lines 5 through 9:

Several different cycling condition occurred to obtain clean PCR product without non-specific amplification. In the beginning a normal cycling condition occurred, containing one initial denaturation at 95 °C for 3 min. followed by 30 cycles of one denaturation at 94 °C for 45 sec, one primer annealing at 60 °C for 45 sec and one extension at 72 °C for one minute. This was followed by a final extension at 72 °C for 5 minutes.

Page 23, lines 14 through 20:

Touch down ~~Down~~ PCR method is an efficient method to eliminate non-specific amplification and thus improves yield and efficiency of a PCR reaction. In touch down ~~Down~~ the annealing of oligonucleotide primer slightly above than the determined (Temperature melting) Tm and the annealing Tm is reduced in each cycle till a desired annealing temperature is achieved. This measure does not allow establishment of non-specific product during the initial cycles and helps improve specific amplification as well as improves efficiency of PCR reaction many folds.

Page 23, lines 22 through 26:

In this program, one initial denaturation at 95 °C for 3 min was followed by 14 touch down ~~Touch Down~~ cycles with one denaturation at 94 °C for 45 Sec, one annealing starting at 70 °C for 45 sec with 0.8 °C decrease in each cycle, one extension at 72 °C for 1 minute. Touch down ~~Down~~ was followed by 25 cycles with one denaturation at 94 °C for 45 Sec, one annealing at 58 °C for 45 Sec and one extension at 72 °C for 1 minute.

Page 23, line 28, through page 24, line 6:

Amplified PCR products were analyzed by electrophoresis on agarose gel. PCR reaction was mixed with 1.0 µl of 6x gel loading buffer and whole reaction was loaded on 1.8 % agarose gel. Gels were prepared and run in 1x TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA. After the electrophoresis, the gel was stained in the staining solution containing 0.5 µg /ml ethidium bromide. ~~Gel~~ Gels were photographed and documented using Eagle eye gel documentation system (Stratagene).

Page 24, lines 9 through 31:

Several method of PCR based detection of mycobacteria in clinical specimens is available as described above. However each method has its own drawbacks and none of them is complete. The main drawback lies in isolation of DNA from clinical specimens. Clinical samples that come to the laboratory for detection contains several impurities that co purify with the DNA by most of the described methods and interfere with further steps of detection. Our method of DNA isolation removes the isolation and provides clean DNA for PCR. Another advantage of this method is selection of oligonucleotide primers for specific amplification of the target DNA stretch specific to pathogenic mycobacteria. A primer set was designed of which one of the primer lies in the intergenic region between an essential gene of mycobacteria while the other is located in an essential gene of mycobacteria. This primer pair is thus very unique to pathogenic mycobacteria and is able to specifically amplify a stretch of DNA from the pathogenic mycobacteria and not from other mycobacteria as is reported for many of the available primers. Other advantage of this method is ~~this do~~ that it does not use expensive reagents for PCR amplification of the target sequence in a stringent condition to achieve specific amplification as this increase the cost of the test. This method instead uses unique cycling condition to achieve specific amplification of the target DNA. So the cost of the test is reduced by approximately 10-20 %. Another advantage of this method is because of the unique structure of the amplified DNA which contain an intergenic region and its flanking region falling in two essential genes of mycobacteria makes this an ideal target for restriction fragment length polymorphism (RFLP) based identification of different strains of pathogenic mycobacteria. Another advantage of this

method is this method incorporates a step in the beginning of the test that inactivates pathogenic mycobacteria in the clinical specimens thus making the process safe for the user.